Comparative toxicity of lead (Pb^{2+}), copper (Cu^{2+}), and mixtures of lead and copper to zebrafish embryos on a microfluidic chip

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Investigations were conducted to determine acute effects of Pb^{2+} and Cu^{2+} presented individually and collectively on zebrafish embryos. Aquatic safety testing requires a cheap, fast, and highly efficient platform for real-time evaluation of single and mixture of metal toxicity. In this study, we have developed a microfluidic system for phenotype-based evaluation of toxic effects of Pb^{2+} and Cu^{2+} using zebrafish (*Danio rerio*) embryos. The microfluidic chip is composed of a disc-shaped concentration gradient generator and 24 culture chambers, which can generate one blank solution, seven mixture concentrations, and eight single concentrations for each metal solution, thus enabling the assessment of zebrafish embryos. To test the accuracy of this new chip platform, we have examined the toxicity and teratogenicity of Pb^{2+} and Cu^{2+} on embryos. The individual and combined impact of Pb^{2+} and Cu^{2+} on zebrafish embryonic development was quantitatively assessed by recording a series of physiological indicators, such as spontaneous motion at 22 hours post fertilization (hpf), mortality at 24 hpf, heartbeat and body length at 96 hpf, etc. It was found that Pb^{2+} or Cu^{2+} could induce deformity and cardiovascular toxicity in zebrafish embryos and the mixture could induce more severe toxicity. This chip is a multiplexed testing apparatus that allows for the examination of toxicity and teratogenicity for substances and it also can be used as a potentially cost-effective and rapid aquatic safety assessment tool. © *2015 AIP Publishing LLC*.
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INTRODUCTION

Industrial and municipal wastewaters and urban storm water commonly contain combinations of metals such as copper, lead, and zinc in addition to organic residues, all of which may be directly or indirectly released into aquatic receiving systems.[1] Contamination of aquatic systems by metals is increasing globally especially in developing countries and represents one of the most critical environmental concerns. As a result, many aquatic environments are contaminated with mixtures containing multiple metals. Lead and copper are two metals that commonly coexist in polluted aquatic environments. Lead is a heavy metal, while copper is an important essential metal. However, both are widespread and highly toxic environmental pollutants.

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Unfortunately, water quality guidelines/criteria that establish limits to chemical releases are usually derived from acute and/or chronic bioassays with individual contaminants and so may fail to predict interactions and associated effects of chemicals in mixture.[2] Concurrent exposure of fish to multiple metals may precipitate metal–metal interactions at uptake sites, binding sites on transport proteins or at cellular target and storage sites.[3]

Despite the fact that lead and copper are found together in many polluted environments, few detailed investigations have examined the combined effects of these two metals. Unlike other aquatic organisms, zebrafish embryos develop externally, and their bodies are transparent enough to characterization under a common optical microscope. More and more people are using zebrafish embryos in metal toxicity studies.[4,5]

For most zebrafish-based metal toxicity analysis experiments, the test platform mainly relied on multi-well plates, beakers[6] or petri dishes.[7] Thus, the embryos were usually exposed statically in culture solution and the solvent would evaporate irreversibly, which resulted in uncontrollable changes of concentration and pH of the original solution. Lammer,[8] Akagi,[9] Choudhury,[10] and Shen[11] established serial microchips for zebrafish embryo culture and toxicity tests, but the chips might only be used for tests of a single drug or metal. Wang[12] designed an integrated microfluidic concentration gradient chip for generating stepwise concentrations in high-density channels and it was applied to high-throughput apoptosis analysis of human uterine cervix cancer (HeLa) cells, but the chip produced a repetitive concentration gradient. Meanwhile, it is very meaningful work that our laboratory reported a new microfluidic system integrated with worm responders for evaluating the environmental manganese toxicity.[13]

Lu[14] described some microfluidic systems in combination with optical methods that have played a great role in the study of *C. elegans*. Herein, we describe a microfluidic device to simultaneously evaluate toxicity and teratogenicity of Pb^{2+} and Cu^{2+} using zebrafish embryos in a real-time imaging manner. Individual and combined concentration of gradient stimulant was generated rapidly through a disc-shaped concentration gradient generator (DCGG) microstructure, which was incorporated into a fish culture room to support dose-dependent drug studies. Upon continuous exposure to zebrafish embryos, a real-time imaging result demonstrates the dose-dependent impairment to organs and tissues during embryogenesis and development. Subtle changes in the transparent embryogenesis can be easily observed and distinguished via common optical microscopes. The fish injury was characterized by spontaneous motion (SM) at 22 hpf, mortality at 24 hpf, heartbeat and body length at 96 hpf, etc. This microfluidic system not only continually delivers test substances but also allows sufficient flowing streams to compensate for the lose of evaporation and to properly remove the harmful excretive waste and metabolite within long periods. Also, the chip can effectively reduce the nonselective adsorption and eliminate evaporation in microchannel area. The method presented here can bridge the gap between animal model and the complex microscale stimulation, which permits a whole-organism-level way to assess the toxicity of individual and two combined metal elements. The microfluidic system could be useful in phenotype-based toxicity evaluation via easy operation, thus holds great promise in high-throughput toxicant and drug screening.

MATERIALS AND METHODS

Materials

All chemicals and reagents were from Aladdin Industrial Corporation (Shanghai, China) unless otherwise stated. Lead acetate (PbAc) and copper sulfate ($CuSO_4$) stock solution of 1.000 g/l were prepared in ultrapure water, respectively, and stored at 4 °C.

Design and fabrication of the microfluidic device

The radial channel network is composed of multi-circle channels and parallel branch channels. In the latitudinal direction of the channel network, three circular channels of 200 μm (width) × 50 μm (depth) are arranged centrically. In the longitudinal direction, the serpentine branch channels of 200 μm (width) × 50 μm (depth) are arranged symmetrically around each of the

circular channels. The solution inlets (1 mm diameter holes) are located inside the first level, and a cylinder-shaped chamber array is located at the downstream of the branch channels in the outermost level. The cylinder-shaped chambers (7 mm in diameter, 2.5 μm in depth) are used for embryo culture. Here, the chip is composed of a DCGG, three liquid inlets, 24 embryo chambers and their inlets and outlets, and 24 liquid outlets. Each circular channel and its surrounding serpentine branch channels are named as a level, and the innermost is the first level (Figs. 1(a) and 1(c)).

The microchannel and culturing chambers were designed using AUTOCAD (2010) software (Fig. S1(A) in Ref. 15) (Autodesk, USA) and converted into a computerized numerical control (CNC) engraving and milling machine (Beijing, China) which has an accuracy of 30 μm. The channel patterns were then scribed into a copper mold (Fig. S1(B) in Ref. 15). The copper-based mold was used for molding polydimethylsiloxane replica (PDMS, Sylgard 184; Dowcorning Corp., USA). The PDMS elastomer base was mixed with curing agent at a 10:1 (w/w) ratio, and degassed at 40 Torr to remove any residual air bubbles. The PDMS mixture was then poured into the copper mold and cured at 65 °C for 2 h. The PDMS wafer was allowed to cool to room temperature and then peeled off from the master mold. In this work, the chip was made of two layers. The top layer (3.5 mm thick), which was made from the structured PDMS slice (Fig. S1(C) in Ref. 15), contained concentration gradient generator (DCGG),

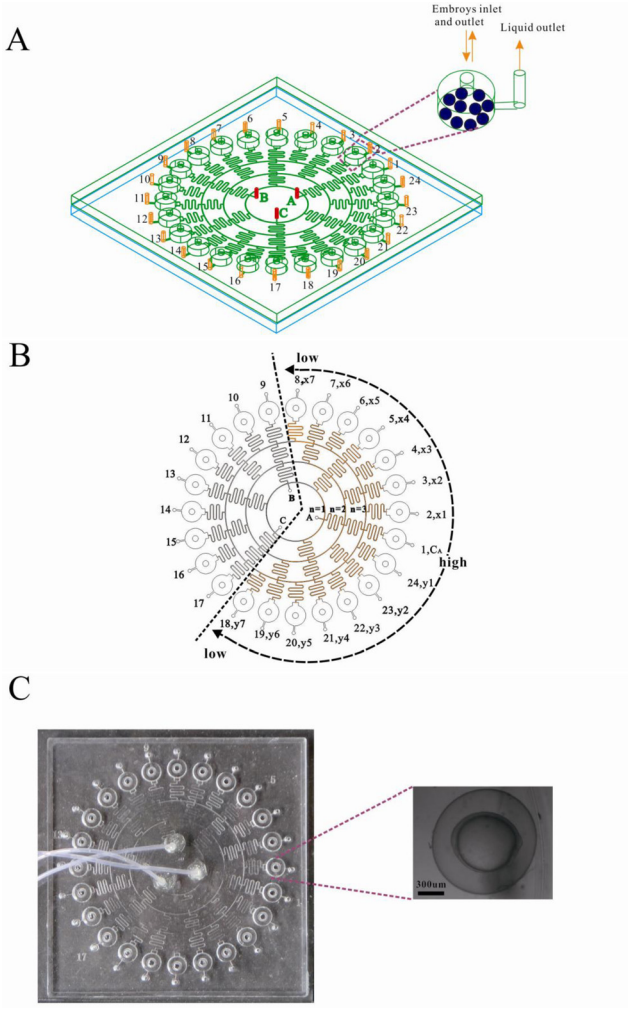

FIG. 1. Embryos on a microfluidic chip. (a) Schematic of chip for zebrafish assay. The chip includes three inlets ((a) is the PbAc inlet, (b) is $CuSO_4$, (c) is ultrapure water), a disc-shaped gradient generator and 24 fish tanks named C1-C24. (b) Schematic of the three-inlet disc-shaped concentration gradient generator. (c) Photos of the microfluidic chip and micrographs of the embryo in the chip.

and each concentration served as embryo culturing chambers (7 mm in diameter, 2.5 mm in height). The bottom layer was made of glass (Fig. S1(D) in Ref. 15, 1.7 mm thick). Then, the two layers were treated by oxygen plasma generator (PDC-32G-2, Harrick, USA), and irreversibly bonded together at 80 °C for 3 h (Fig. S1(E) in Ref. 15).

The culturing medium and metal solution were delivered into the device by a syringe pump (LSP10–10B, LongerPump, Baoding, China), via Teflon tubes (ID 0.5 mm, OD 1.0 mm) connected to the inlets and outlets of the microfluidic chip.

Concentration gradient generation and characterization

The fluorescent dye solutions (Eosin Y in A, FITC in B, and ethanol in C) were simultaneously introduced into the channel network at the same flow rate by syringe pumps. Each source solution split into three branch streams when entering the circular channel. Half of the solution directly entered the branch channel just opposite and remained at the original concentration. Two quarters of the solution flowed into the two adjacent branches, where they met and mixed with one quarter stream of two adjacent source solutions respectively. Wang[12] and Zhang[13] described the solution flowing and the concentration gradient generating in considerable detail in such type of chip. The two dye solutions and the ethanol were disturbed three times and collected through the liquid outlets. The fluorescent intensity was determined by automatic micro plate reader.

Fish maintenance and embryos collected

The wild-type zebrafish (provided by School of Life Sciences, Sun Yat-sen University, China) were housed in a ratio of 2 females to 1 male in a stand-alone aquarium (L × B × H, 10 cm × 20 cm × 10 cm) with a flow-through system.[16] The fish were kept on a 14 h light/10 h dark circle and the water temperature was set at 26 ± 1 °C.[17] Constant filtering or constant flow-through conditions ensured that ammonia, nitrite, and nitrate were kept below the detection limits, which were 0–5, 0.025–1, and 0–140 mg l^{-1}, respectively.[8] The zebrafish were fed with a commercially available artificial diet (*Paramecium*) twice-a-day, while overfeeding was strictly avoided to ensure optimal water quality. Uneaten food and feces were removed daily.

Males and egg-laden females were housed separately but were placed in the same tank for one night during spawning. Embryos were obtained by spawning six to ten groups of genitors with a male:female ratio of 2:1. We placed the fish in a specific spawning aquarium at 28 °C with a mesh bottom to protect the eggs from being eaten. Spawning and fertilization took place within 30 min when the light was switched on in the morning. Then, the embryos of zebrafish were collected in a sedimentation tank using purpose-built egg traps. The newly fertilized eggs were transferred into petri dishes filled with culture water. Note that the ultrapure water did not contain methylene blue or antibiotics and was rinsed to remove debris and dead embryos every 30 min.[18] The normal and healthy developed eggs at different developmental stages were then selected by using a sterile micropipette under a stereomicroscope (SMZ-T4, Optec, Chongqing, China) at 3 hpf (Ref. 19) and cleaned twice with ultrapure water[20] for the subsequent microfluidic application. Ultrapure water medium was aerated for at least half an hour before addition of the test chemicals.[21]

Continuous metal treatment of zebrafish embryos in a microfluidic device

Freshly spawned eggs can be recognized by a fully transparent perivitelline space surrounded by the egg membrane, the yolk, and the germinal disc that forms at the animal pole. Only fertilized eggs were used for analysis while embryos with overt anomalies (asymmetries, formation of vesicles) or damaged membranes were discarded. Non-fertilized eggs can be identified by the lack of blastomeric formation and, at later stages, by their lack of transparency.

The microfluidic device was rinsed with 0.1 mol l^{-1} HNO_3, 0.1 mol l^{-1} NaOH, and ultrapure water, in that order. Then, the selected zebrafish embryos were transferred into the chamber by a modified pipette tip,[22] and then sedimented into the cylinder-shaped chambers. For the

microfluidic chip embryo toxicity test, 10–12 fresh zebrafish eggs (3 hpf) were selected and transferred into the chamber. To facilitate the trapping process, each culture compartment was prefilled with ultrapure water. The waste solution was discharged via the liquid outlets. In this experiment, the inlet A with $1\,mg\,l^{-1}$ PbAc (refer to Chen[23]), the B inlet with $0.1\,mg\,l^{-1}$ $CuSO_4$ (refer to Liu[24]) solution, which was diluted from the stock solution of $1\,g\,l^{-1}$, respectively.

The flow stream was created by a syringe pump with positive pressure with a flow rate of $10\,\mu l$/min and the microchip was maintained in a custom-designed incubator at 28.5 °C to ensure the normal development of the embryos. After 48 h treatment, the test chemicals were replaced by the ultrapure water, and the embryos were observed directly under a microscope (SMZ-T4, Optec, Chongqing, China). All of the experimental procedures were carried out in accordance with the China Animal Welfare Legislation, and were approved by the ethics committee on the Care and Use of Laboratory Animals at Sun Yat-sen University, Guangzhou, People's Republic of China.

Assessment of toxicity and teratogenicity

The microscale networks provided gradient metal and metal-metal solutions to stimulate the eggs and generate toxic and teratogenic impairments. Based on well-documented morphology at a variety of developmental stages,[25] a physiologically precise evaluation regarding the toxicity and teratogenicity on the embryos can be carried out by morphological analysis. At different growth stages, varied representative signs were able to be recorded, such as heart rate, pericardium and yolk shape, spontaneous motion or body twisting, number of somites, the development of eyes, notochord and body shape, tail fin size, pigmentation, and so on. The toxic assessments were mainly characterized with developmental arrest, spontaneous movements, and heart rates.

SM at 22 hpf, body length and heartbeat at 96 hpf,[23] mortality at 24 hpf,[3] and hatching rate at 72 hpf are very important representative signs (evaluating points) in our toxicity test, which are directly counted by the naked eye through a microscope to get physiologically real cardiac activity.

Image and data analysis

To achieve a clear verification of the toxic impact of the test chemicals on the embryonic development, the endpoints were documented by employing an inverted optical microscope (4×, 10× objective, BDS200, Optec, Chongqing, China) with a digital camera (DM200). We selected heart rate, body twisting, pigmentation, tail detachment, development of eyes, segmentation, and teratogenicity as evaluating endpoints. The recorded photographs were analyzed using OPTPRO software. To get comprehensive and detailed information on the deformations caused by the chemical treatment, microscope with 10× objective was used to obtain the target feature.

The data presented in this study were checked for normality and homogeneity of variances by the Kolmogorov-Smirnov one-sample test and the Levene test. The differences between groups were calculated with a one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test using the SPSS 11.0 software (IBM, Statistical Product and Service Solutions, Version 11.0) with a p value of $p < 0.05$ and $p < 0.01$ considered to be statistically significant. The figures were drawn using Origin 8.0 (OriginLab, Northampton, MA, USA).

Spontaneous movement analysis and body length measured

SM is an important parameter in small animal experiments. Roy Lycke[26] invented a microfluidics chip to identify modes of *Caenorhabditis elegans* paralysis in four anthelmintics by recording the curls per second, types of paralyzation, mode frequency, and number/duration of active/immobilization periods. SM represents the first motor activity generated by the developing motor network in zebrafish. Spontaneous movement of embryos (alternating tail bending or coiling) was recorded using a CCD camera (Optec, Chongqing, China) mounted on a microscope at 22 hpf refer to Chen[23] research. To determine whether PbAc and $CuSO_4$ exposure affects SM, five embryos per chamber were performed for date analysis.

A microsurgery needle was employed to pierce the embryonic membrane carefully to help the embryos hatch under a microscope when the zebrafish embryo could not be hatched at 84 hpf. Body length of larvae was measured except the ones curved severely.

RESULTS AND DISCUSSION

Considerations and characterization of the chip

In this chip, all the gradients were formed with the prerequisites of complete mixing. The microchannel network was designed into a circular shape, making utmost use of the space of the chip and allowing embryo chambers to be arranged at the outermost region of the network. The mixture of fluids in the serpentine branch channels in the longitudinal direction and their circulation in the latitudinal direction were described in Fig. 1(b).

As shown in Fig. 1(b), the inner circular shape is $n = 1$, the middle circular is $n = 2$, and the outmost one is $n = 3$. The total number of chambers is 3×2^n. The number of the chambers which containing one of inlet solutions is $2^{n+1} - 1$, for example, due to $n = 3$ in this chip, the number of chambers containing substance A is 15 ($2^{3+1} - 1$), they are chambers 1, 2, 3, 4, 5, 6, 7, 8, 18, 19, 20, 21, 22, 23, and 24, respectively. In this chip, the concentrations in the left and the right downstream channels are both 1/2 of the upstream concentration, thus the concentrations in the chambers can be easily estimated by the code number of each branch channel. In the case of substance A, $xi = [(2^n - i)C_A + iC_B]/2^n$,$yi = [(2^n - i)C_A + iC_C]/2^n$ ($1 \le i \le 2^n - 1$). We can infer from the formula that the concentration including substance A decreasing form C_A to x7 and y7.

In order to further characterize the concentration gradient of the chip. The concentration gradient generated by the fluorescent dye (inlet A is Eosin Y, inlet B is FITC) solutions was gathered and determined by automatic microplate reader (FLX Station3, Molecular Devices Corporation, USA). The fluorescent intensity in the chambers was recorded at Fig. 2.

Fig. 2 shows that the fluorescent dye formed the concentration gradients are closed to the theoretical value when the flow rate greater than 5 μl/min. The correlation coefficients are greater than 0.90.

From Fig. 2 and the above analysis we can infer the concentration change trend of Pb^{2+} and Cu^{2+} in the chip. The 24 culture chambers were divided into three zones including metal element and blank control. The mixture zones include C2 to C8, and the concentration of Pb^{2+} is lower and lower in contrast to Cu^{2+} from C2 to C8. The trends of concentration in signal zone and the control group are shown in Fig. 3.

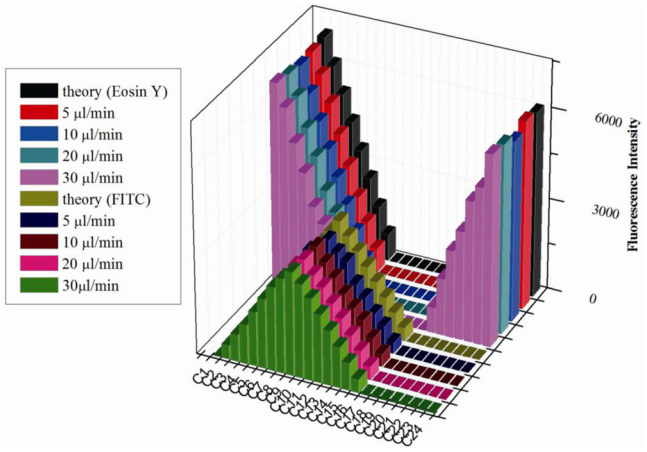

FIG. 2. Quantitative comparison of the formed chemical concentrations in the terminal chambers between the actual and theoretical value. The Eosin Y (inlet A), FITC (inlet B), and E3 (inlet C) are distributed by the chip at different rate ($n = 3$), correlation coefficient (r) is compared with the theory group.

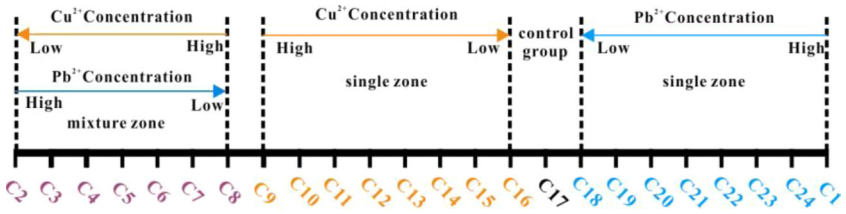

FIG. 3. Concentration change trend of Pb^{2+} and Cu^{2+} in the chip.

Embryo culturing on chips

To obtain a reliable and precise metal toxicity evaluation of the chip for zebrafish embryos, we examined the development and growth of embryos in chip culture chambers by exposure to blank solution. Zebrafish embryos (3 hpf) were dispensed into the culture rooms (10 eggs per room) and multiple flow rates ranging from 5 μl/min to 30 μl/min at each inlet were examined on their development. For comparison, zebrafish embryos (3 hpf) were also cultured in 24-well plate (10 per well) with periodic static and renewal blank solution every 24 h as controls. The mortality rate was the critical indicator for estimating the development state of zebrafish embryos.

During the 84 hpf culture period, a time-dependent imaging of fish body and organs was taken to record developmental stages every 12 hpf. It was observed that all of the surviving embryos hatched successfully with full-grown organs and the larvae have already shown a complex behavioral repertoire. The zebrafish embryos developing in the chip exhibit lower mortality than the ones cultured in static 24-micro-well with periodic blank solution (Fig. 4). Mortality of zebrafish embryos did not change at 24 hpf; we evaluated metal element toxicity using the mortality of 24 hpf.

Pb^{2+} and Cu^{2+} affect SM at 22 hpf and mortality at 24 hpf

The average tail bend frequency of the control (the 17 chamber) was 9.69 ± 1.63 bents/min at 22 hpf ($n = 5$). Exposure to PbAc or $CuSO_4$ caused significant decrease in tail bent frequency compared to the control, but there is no significant difference at the low concentration in chamber 16 and chamber 18. In addition, SM in mixed area (C2 to C8) has significant difference with the control chamber (Fig. 5(a)).

The average mortality of the control (the 17 chamber) was $8.3 \pm 2.9\%$ at 24 hpf ($n = 3$). The mortality demonstrated concentration-dependence in single Pb^{2+} (concentration from high to low

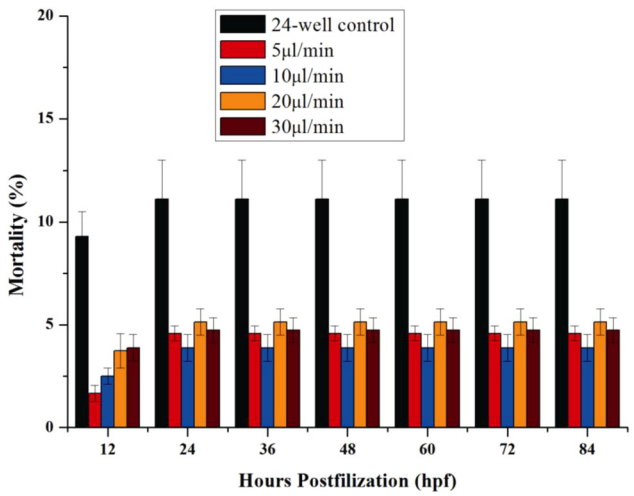

FIG. 4. Mortality of zebrafish embryos in microfluidic chip under blank flowing stream.

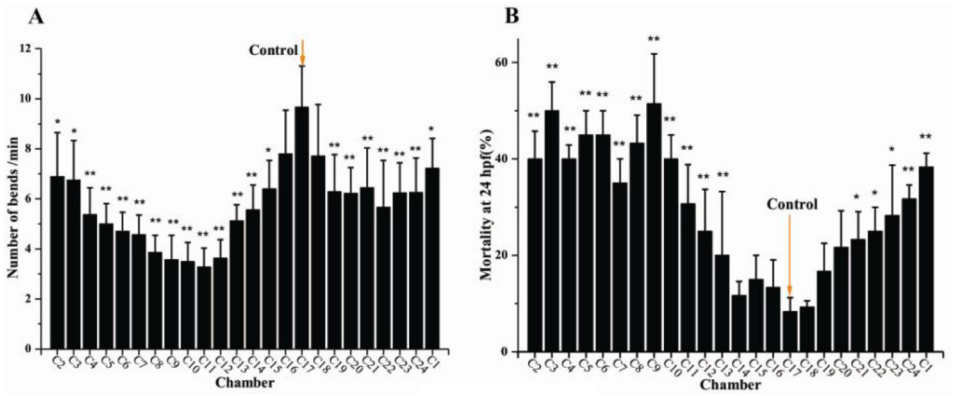

FIG. 5. Impact of PbAc and $CuSO_4$ exposure to zebrafish embryos. (a) Embryos were exposed to the metal solution from 3 hpf. Numbers of bends per min were record at 22 hpf (n = 5). (b) Mortality of the embryos exposed to the PbAc and $CuSO_4$ at 24 hpf (n = 3). (Average ± standard deviation; analysis of variance, Dennett's test; *: significant difference compared to the controls at $p < 0.05$, **: significant difference compared to the controls at $p < 0.01$).

of C1, C24, C23, C22, C21, C20, C19, and C18 in turn) or Cu^{2+} (concentration from high to low of C9, C10, C11, C12, C13, C14, C15, and C16 in turn) solution, but there is no significant difference at the low concentration (Pb^{2+}, 18, 19, 20; Cu, 14, 15, 16). The mortality of mixture solution (Pb^{2+} and Cu^{2+} (C2-C8)) was obviously higher than the control group (Fig. 5(b)). The average mortality of zebrafish in our chip is consistent with the results on the 24-well plastic plates.

Pb^{2+} and Cu^{2+} affect the heartbeat and the bodylength at 96 hpf

The average rate of heartbeat of zebrafish in the control chamber was 144 ± 16.5/min. There are significant differences in the effect on zebrafish heart rate in single Pb^{2+}, single Cu^{2+} and a mixture of the two metal elements. The heartbeat of larvae in mixture zone (C2-C8) was lower than the ones in single metal element except C1 (Fig. 6(a)). With lower heartbeat, the greater cardiac toxicity of metal can be observed, while the mixture metal solution and high concentration of Pb^{2+} have serious toxicity to 96 hpf zebrafish.

Body length is one of the important indexes in zebrafish development. The lengths of zebrafish in high concentrations of Pb^{2+} (C1, C24, C23), Cu^{2+} (C9) and mixture metal groups have significant differences in comparison to the control group (C17). However, low concentrations of Pb^{2+} (C18-C22) or Cu^{2+} (C10-C16) show no significant differences in comparison to C17 (Fig. 6(b)). The average of zebrafish heartbeat and body length of zebrafish at 96 hpf in chip have no significant difference compared to the embryos cultured in a 24-well plastic plate in the same concentration of Cu^{2+} and Pb^{2+}.

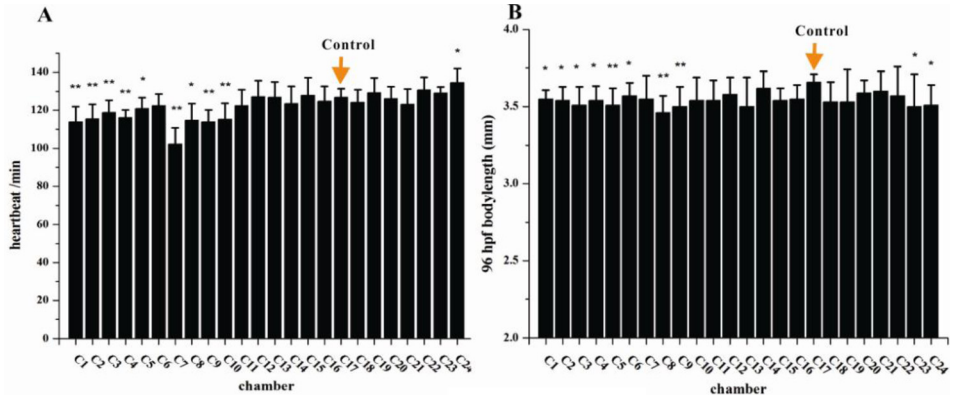

FIG. 6. Bodylength and heartbeat of zebrafish at 96 hpf. (a) Heartbeat of zebrafish at 96 hpf (n = 5); (b) length of zebrafish at 96 hpf (n = 5). (Average ± standard deviation; analysis of variance, Dennett's test; *: significant difference compared to the controls at $p < 0.05$, **: significant difference compared to the controls at $p < 0.01$).

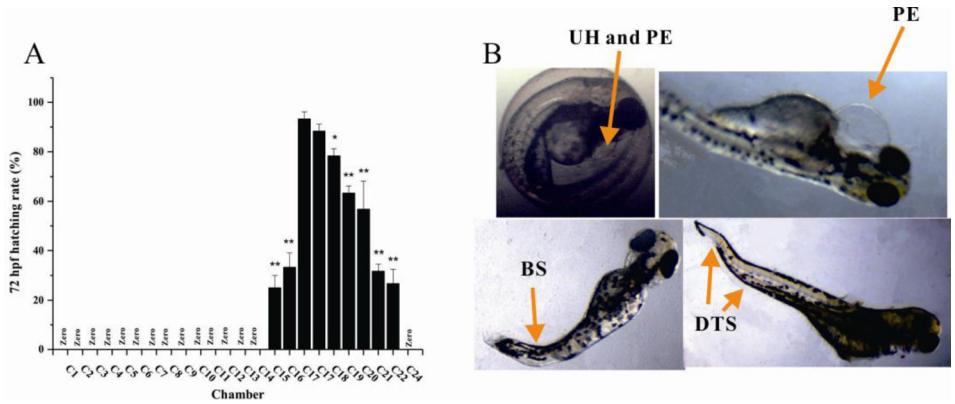

FIG. 7. Pb^{2+} and Cu^{2+} induced abnormal morphology, developmental retardation, and mortality of zebrafish embryos. (a) Hatched rate at 72 h. (b) Typical morphological abnormalities of embryos exposed to Pb^{2+} and Cu^{2+}. BS: bent spine, PE: pericardial edema, DTS: double tail spine, UH: Unhatched. (Average ± standard deviation; *: significant difference compared to the controls at $p < 0.05$, **: significant difference compared to the controls at $p < 0.01$).

Hatching and teratogenicity of Pb^{2+} and Cu^{2+} on zebrafish embryos

The hatching of zebrafish embryos was strongly inhibited in high concentrations of single Pb^{2+} (C1, C24) or single Cu^{2+} (C9, C10, C11, C12, C13, C14), while low concentration of single metal (Pb^{2+}, C18, C19, C20, C21, C22; Cu^{2+}, C15, C16) has a weak influence on hatching, Fig. 7(a). The results are very close to that of the embryos cultured in a 24-well plastic plate under the same conditions.

The adverse effects of zebrafish embryos were analyzed at 96 hpf following Pb^{2+} and Cu^{2+} exposure from 3 hpf to 48 hpf, and then cultured with ultrapure water rich in oxygen after 48 hpf. The present study displayed the teratogenicity of Pb^{2+} and Cu^{2+} on zebrafish embryos, including bent spine, double tail spine, pericardial edema, and yolk sac edema. Several kinds of malformations often appeared together in one larva (Fig. 7(b)).

CONCLUSION

Microfluidic technology has become an attractive platform for analysis of biological phenomena.[27,28] In this study, we developed a microfluidic system to evaluate the dynamic developmental toxicity and teratogenicity of single and combined Pb^{2+} and Cu^{2+} on zebrafish embryos. The microfluidic device offers three inlets for Pb^{2+}, Cu^{2+}, and culture solution, respectively. The chip includes a disc-shaped DCGG, 24 egg culture chamber, 3 solution inlets, and 24 waste outlets. The embryos can be developed normally in microscale tanks with open structures to remove dead embryos immediately. Dynamic development in the presence or absence of Pb^{2+} and Cu^{2+} can be easily characterized by common optical imaging in a real-time manner. This was shown as delayed development, bent spine, double tail spine, pericardial edema, and yolk sac edema malformations. This innovative microsystem has great potential to compete with a microplate platform to perform metal safety evaluations and poison screening using embryos as vertebrate models, especially at the single organism level.

ACKNOWLEDGMENTS

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[1]N. L. Cooper, J. R. Bidwell, and A. Kumar, "Toxicity of copper, lead, and zinc mixtures to *Ceriodaphnia dubia* and *Daphnia carinata*," Ecotoxicol. Environ. Saf. **72**(5), 1523–1528 (2009).
[2]J. R. Shaw, T. D. Dempsey, C. Y. Chen, J. W. Hamilton, and C. L. Folt, "Comparative toxicity of cadmium, zinc, and mixtures of cadmium and zinc to daphnids," Environ. Toxicol. Chem. **25**(1), 182–189 (2006).

[3]C. Kamunde and R. MacPhail, "Metal-metal interactions of dietary cadmium, copper and zinc in rainbow trout, *Oncorhynchus mykiss*," Ecotoxicol. Environ. Saf. **74**(4), 658–667 (2011).
[4]P. V. Asharani, Y. L. Wu, Z. Y. Gong, and S. Valiyaveettil, "Toxicity of silver nanoparticles in zebrafish models," Nanotechnology **19**(25), 255102 (2008).
[5]K. Augustine-Rauch, C. X. Zhang, and J. M. Panzica-Kelly, "*In vitro* developmental toxicology assays: A review of the state of the science of rodent and zebrafish whole embryo culture and embryonic stem cell assays," Birth Defects Res., Part C **90**(2), 87–98 (2010); N. N. He, X. Li, D. F. Feng, M. Wu, R. Chen, T. H. Chen, D. Y. Chen, and X. Z. Feng, "Exploring the toxicity of a bismuth-asparagine coordination polymer on the early development of zebrafish embryos," Chem. Res. Toxicol. **26**(1), 89–95 (2013); Y. Pan, A. Leifert, M. Graf, F. Schiefer, S. Thoroe-Boveleth, J. Broda, M. C. Halloran, H. Hollert, D. Laaf, U. Simon, and W. Jahnen-Dechent, "High-sensitivity real-time analysis of nanoparticle toxicity in green fluorescent protein-expressing zebrafish," Small **9**(6), 863–869 (2013).
[6]A. Johnson, E. Carew, and K. A. Sloman, "The effects of copper on the morphological and functional development of zebrafish embryos," Aquat. Toxicol. **84**(4), 431–438 (2007).
[7]M. Riggio, S. Filosa, E. Parisi, and R. Scudiero, "Changes in zinc, copper and metallothionein contents during oocyte growth and early development of the teleost *Danio rerio* (zebrafish)," Comp. Biochem. Physiol. C: Toxicol. Pharmacol. **135**(2), 191–196 (2003); S. S. Lewis and S. J. Keller, "Identification of copper-responsive genes in an early life stage of the fathead minnow *Pimephales promelas*," Ecotoxicology **18**(3), 281–292 (2009); C. E. Leite, L. D. Maboni, F. F. Cruz, D. B. Rosemberg, F. F. Zimmermann, T. C. B. Pereira, M. R. Bogo, C. D. Bonan, M. M. Campos, F. B. Morrone, and A. M. O. Battastini, "Involvement of purinergic system in inflammation and toxicity induced by copper in zebrafish larvae," Toxicol. Appl. Pharmacol. **272**(3), 681–689 (2013).
[8]E. Lammer, H. G. Kamp, V. Hisgen, M. Koch, D. Reinhard, E. R. Salinas, K. Wendler, S. Zok, and T. Braunbeck, "Development of a flow-through system for the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*)," Toxicol. in Vitro **23**, 1436–1442 (2009).
[9]J. Akagi, K. Khoshmanesh, B. Evans, C. J. Hall, K. E. Crosier, J. M. Cooper, P. S. Crosier, and D. Wlodkowic, "Miniaturized embryo array for automated trapping, immobilization and microperfusion of zebrafish embryos," PLoS ONE **7**(5), e36630 (2012).
[10]D. Choudhury, D. van Noort, C. Iliescu, B. X. Zheng, K. L. Poon, S. Korzh, V. Korzh, and H. Yu, "Fish and Chips: A microfluidic perfusion platform for monitoring zebrafish development," Lab Chip **12**(5), 892–900 (2012).
[11]Y. C. Shen, D. Li, A. Al-Shoaibi, T. Bersano-Begey, H. Chen, S. Ali, B. Flak, C. Perrin, M. Winslow, H. Shah, P. Ramamurthy, R. H. Schmedlen, S. Takayama, and K. F. Barald, "A student team in a University of Michigan biomedical engineering design course constructs a microfluidic bioreactor for studies of zebrafish development," Zebrafish **6**(2), 201–213 (2009).
[12]C. G. Yang, Y. F. Wu, Z. R. Xu, and J. H. Wang, "A radial microfluidic concentration gradient generator with high-density channels for cell apoptosis assay," Lab Chip **11**(19), 3305–3312 (2011).
[13]B. B. Zhang, Y. B. Li, Q. D. He, J. Qin, Y. Y. Yu, X. C. Li, L. Zhang, M. C. Yao, J. S. Liu, and Z. G. Chen, "Microfluidic platform integrated with worm-counting setup for assessing manganese toxicity," Biomicrofluidics **8**, 054110 (2014).
[14]G. Aubry and H. Lu, "A perspective on optical developments in microfluidic platforms for *Caenorhabditis elegans* research," Biomicrofluidics **8**, 011301 (2014).
[15]See supplementary material at http://dx.doi.org/10.1063/1.4913699 for details on the fabrication process of an integrated microfluidic chip for embryonic zebrafish assay.
[16]E. Teixido, E. Pique, J. Gomez-Catalan, and J. M. Llobet, "Assessment of developmental delay in the zebrafish embryo teratogenicity assay," Toxicol. in Vitro **27**(1), 469–478 (2013).
[17]F. Yang, Z. G. Chen, J. B. Pan, X. C. Li, J. Feng, and H. Yang, "An integrated microfluidic array system for evaluating toxicity and teratogenicity of drugs on embryonic zebrafish developmental dynamics," Biomicrofluidics **5**(2), 24115 (2011).
[18]E. M. Wielhouwer, S. Ali, A. Al-Afandi, M. T. Blom, M. B. O. Riekerink, C. Poelma, J. Westerweel, J. Oonk, E. X. Vrouwe, W. Buesink, H. G. J. vanMil, J. Chicken, R. van't Oever, and M. K. Richardson, "Zebrafish embryo development in a microfluidic flow-through system," Lab Chip **11**(10), 1815–1824 (2011).
[19]T. S. Wu, J. J. Yang, F. Y. Yu, and B. H. Liu, "Evaluation of nephrotoxic effects of mycotoxins, citrinin and patulin, on zebrafish (*Danio rerio*) embryos," Food Chem. Toxicol. **50**(12), 4398–4404 (2012).
[20]S. Weigt, N. Huebler, R. Strecker, T. Braunbeck, and T. H. Broschard, "Zebrafish (*Danio rerio*) embryos as a model for testing proteratogens," Toxicology **281**(1–3), 25–36 (2011).
[21]G. Carlsson, J. Patring, J. Kieuger, L. Norrgren, and A. Oskarsson, "Toxicity of 15 veterinary pharmaceuticals in zebrafish (*Danio rerio*) embryos," Aquat. Toxicol. **126**, 30–41 (2013).
[22]Y. B. Li, F. Yang, Z. G. Chen, L. J. Shi, B. B. Zhang, J. B. Pan, X. C. Li, D. P. Sun, and H. Z. Yang, "Zebrafish on a chip: A novel platform for real-time monitoring of drug-induced developmental toxicity," PLoS ONE **9**(4), e94792 (2014).
[23]J. F. Chen, Y. H. Chen, W. Liu, C. L. Bai, X. X. Liu, K. Liu, R. Li, J. H. Zhu, and C. J. Huang, "Developmental lead acetate exposure induces embryonic toxicity and memory deficit in adult zebrafish," Neurotoxicol. Teratol. **34**(6), 581–586 (2012).
[24]G. X. Liu, X. L. Chai, Y. Q. Shao, L. H. Hu, Q. L. Xie, and H. X. Wu, "Toxicity of copper, lead, and cadmium on the motility of two marine microalgae *Isochrysis galbana* and *Tetraselmis chui*," J. Environ. Sci. **23**(2), 330–335 (2011).
[25]C. B. Kimmel, W. W. Ballard, S. R. Kimmel, B. Ullmann, and T. F. Schilling, "Stages of embryonic development of the zebrafish," Dev. Dyn. **203**, 253–310 (1995).
[26]R. Lycke, A. Parashar, and S. Pandey, "Microfluidics-enabled method to identify modes of *Caenorhabditis elegans* paralysis in four anthelmintics," Biomicrofluidics **7**, 064103 (2013).
[27]H. Huang, L. Jiang, S. Li, J. Deng, Y. Li, J. Yao, B. Li, and J. Zheng, "Using microfluidic chip to form brain-derived neurotrophic factor concentration gradient for studying neuron axon guidance," Biomicrofluidics **8**, 014108 (2014).
[28]O. Moreno-Arotzena, G. Mendoza, M. Cóndor, T. Rüberg, and J. M. García-Aznar, "Inducing chemotactic and haptotactic cues in microfluidic devices for three-dimensional *in vitro* assays," Biomicrofluidics **8**, 064122 (2014).